Current Biology, Vol. 14, 618–624, April 6, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.02.042

# Two Modes by which Lefty Proteins Inhibit Nodal Signaling

**Canhe Chen and Michael M. Shen***
Center for Advanced Biotechnology and Medicine and Department of Pediatrics
University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School
Piscataway, New Jersey 08854

## Summary

**During vertebrate embryogenesis, members of the Lefty subclass of Transforming Growth Factor-β (TGFβ) proteins act as extracellular antagonists of the signaling pathway for Nodal, a TGFβ-related ligand essential for mesendoderm formation and left-right patterning [1–3]. Genetic and biochemical analyses have shown that Nodal signaling is mediated by activin receptors but also requires EGF-CFC coreceptors, such as mammalian Cripto or Cryptic [4–8]. Misexpression experiments in zebrafish and frogs have suggested that Lefty proteins can act as long-range inhibitors for Nodal, possibly through competition for binding to activin receptors [9–13]. Here we demonstrate two distinct and unexpected mechanisms by which Lefty proteins can antagonize Nodal activity. In particular, using a novel assay for Lefty activity in mammalian cell culture, we find that Lefty can inhibit signaling by Nodal but not by Activin or TGFβ1, which are EGF-CFC independent. We show that Lefty can interact with Nodal in solution and thereby block Nodal from binding to activin receptors. Furthermore, Lefty can also interact with EGF-CFC proteins and prevent their ability to form part of a Nodal receptor complex. Our results provide mechanistic insights into how Lefty proteins can achieve efficient and stringent regulation of a potent signaling factor.**

## Results and Discussion

### Inhibition of Nodal Signaling by Lefty Proteins in Cell Culture

Lefty proteins are atypical members of the TGFβ family because they lack an α-helix and a crucial cysteine residue essential for formation of homo- or heterodimers and their covalent stabilization [9, 14, 15]. To establish an assay for Lefty activity, we first expressed epitope-tagged mouse Lefty1 protein in human 293T cells and found that it could be readily secreted into the culture medium. Under these conditions, a significant proportion of both Nodal and Lefty1 proproteins can undergo cleavage to release mature proteins of the expected size (Figure 1A).

Previously, we have described a cell culture assay for Nodal signaling in which Nodal activity is dependent upon expression of an EGF-CFC protein and the transcription factor FoxH1 (FAST2) [8, 16]. We have now modified this assay to assess Lefty activity by expressing Nodal, Cripto, and FoxH1 in 293T cells in the absence or presence of cotransfected Lefty1 and by quantifying Nodal activity by using the Nodal/Activin-responsive luciferase reporter *A3-luc* (Figure 1B, left). We have found that Lefty1 could strongly inhibit Nodal signaling as measured by luciferase activity (Figure 1C); similar results were obtained in parallel experiments using Lefty2 (data not shown). To show that Lefty protein can act in trans as a soluble factor, we determined whether Lefty1 protein present in conditioned media from transfected 293T cells could antagonize Nodal activity from conditioned media of independently transfected 293T cells (Figure 1B, right). We found that increasing amounts of Lefty1-conditioned media could progressively inhibit exogenous Nodal activity (Figure 1D), indicating that Lefty can indeed act as a soluble factor, consistent with its ability to act non-cell-autonomously in vivo [11, 17]. In addition, because Nodal signaling requires the presence of EGF-CFC proteins [5, 6, 8, 18], we asked whether the inhibitory activity of Lefty protein would function for all EGF-CFC proteins or whether it was specific for Cripto. We found that both mouse Cryptic and zebrafish Oep would stimulate Nodal signaling in this assay, as previously reported [8], and that this signaling activity could be blocked in each case by Lefty1 (Figure 1E).

We next addressed whether Lefty1 inhibitory activity was specific for Nodal or whether it could antagonize other TGFβ factors. We found that Lefty1 could also block the signaling activity of *Xenopus* Vg1 (Figure 1C), which utilizes activin receptors and EGF-CFC coreceptors similarly to Nodal [19]. In contrast, Lefty1 was unable to inhibit signaling by Activin A or by TGFβ1 (Figures 1F and 1G), which are both EGF-CFC independent; similar results were obtained for Lefty2 (data not shown). Although Lefty has also been named "Antivin" because of the mutually antagonistic effects of Activin and Lefty misexpression in fish and frog embryos [9, 10, 20, 21], our cell culture results suggest that Lefty does not directly block Activin signaling. We are uncertain as to the basis for this discrepancy, but one possible explanation for Lefty's ability to suppress the effects of Activin misexpression in vivo is that it could inhibit a Nodal positive-feedback loop induced by ectopic Activin. Conversely, Activin may suppress the Lefty misexpression phenotype because of its ability to stimulate the Nodal signaling pathway in a Lefty- and EGF-CFC-independent manner. However, we note that the previous finding that Lefty can inhibit Vg1 activity in vivo [22] is consistent with the role of EGF-CFC proteins in mediating Vg1 signaling activity [19].

### Two Modes of Lefty1 Inhibition of Nodal Receptor Complex Formation

To investigate the molecular basis by which Lefty proteins can antagonize Nodal signaling, we examined whether Lefty could inhibit the formation of a Nodal receptor complex. Previous studies have shown that

*Correspondence: mshen@cabm.rutgers.edu

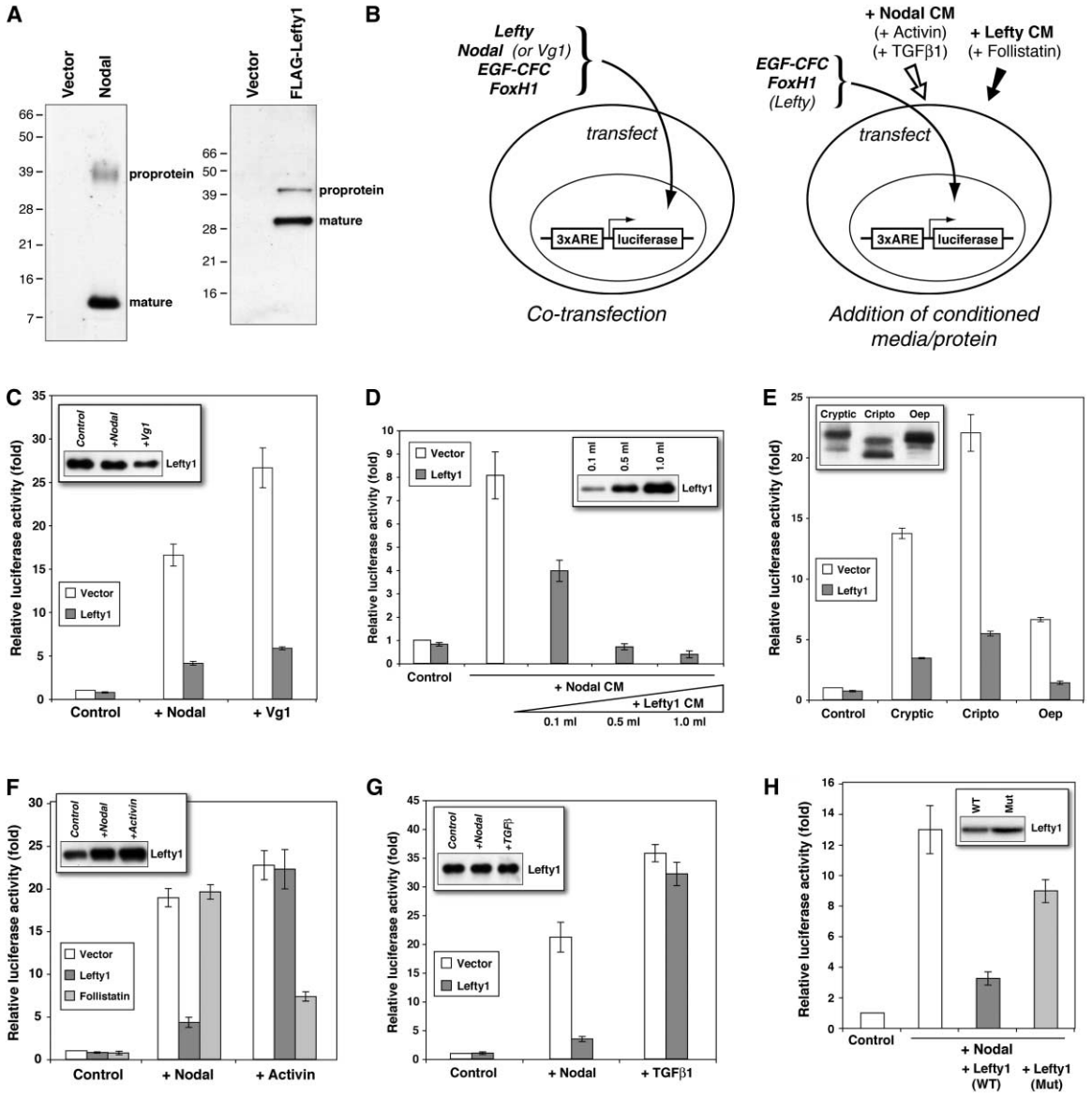


Figure 1. Lefty Proteins Inhibit the Activity of Nodal and Vg1, but Not Activin or TGFβ1

(A) Expression of mouse Nodal and Lefty1 proteins in culture supernatants of transfected 293T cells. The observed size of the epitope-tagged Lefty1 mature protein (30 kDa) is consistent with prodomain cleavage occurring primarily at the second predicted processing site (RQKR, amino acids 132–135). Positions of molecular standards are indicated. (B) Schematic design of assays for Lefty activity. (Left) 293T cells are transfected with expression constructs for Lefty, Nodal (or Vg1), an EGF-CFC protein, and the FoxH1 transcription factor, together with the Nodal/Activin-responsive *A3-luc* reporter (C, E, and H). (Right) 293T cells are transfected with expression constructs for an EGF-CFC protein and FoxH1 and treated with conditioned media containing Nodal and/or conditioned media containing Lefty (D); alternatively, an expression construct for Lefty is cotransfected, and recombinant Activin A, TGFβ1, and/or Follistatin protein is added (F and G). In all assays, cotransfection of an expression construct for β-galactosidase was used to normalize for transfection efficiency, and addition of empty *pcDNA3* vector was used to maintain a constant amount of transfected DNA. (C) Nodal activity is inhibited by Lefty1 in the presence of cotransfected Cripto and FoxH1; control samples correspond to cells transfected with Cripto and FoxH1 without Nodal. Lefty1 similarly inhibits the activity of *Xenopus* Vg1, expressed using a heterologous BMP2 prodomain fusion to facilitate processing [32]. Inset: Western blot detection of mature Lefty1 protein (approximately 30 kDa) in culture supernatants. (D) Inhibition of Nodal activity in conditioned media by a dilution series of Lefty1-conditioned media was assayed on cells transfected with expression constructs for Cripto and FoxH1; controls correspond to these cells with the addition of conditioned media from cells transfected with empty vector or with Lefty1. Inset: Western blot detection of mature Lefty1 protein (approximately 30 kDa) in culture supernatants. (E) Lefty1 can inhibit Nodal activity in the presence of FoxH1 and cotransfected Cryptic, Cripto, or Oep; control samples correspond to cells transfected with Nodal and FoxH1 without EGF-CFC proteins. Inset: α-FLAG antibodies (FLAG-Cryptic, approximately 22 and 20 kDa; FLAG-Cripto, approximately 21 and 19 kDa; FLAG-Oep, approximately 21 kDa) were used for Western blot detection of EGF-CFC proteins in cell lysates. (F) Transfected Lefty1 does not inhibit activity of exogenous recombinant Activin A, whereas recombinant Follistatin can block Activin signaling but does not affect Nodal. All samples were cotransfected with Cripto and FoxH1; controls correspond to these cells cotransfected with vector or Lefty1 or to these cells with the addition of Follistatin. Inset: Western blot of mature Lefty1 protein in culture supernatants. (G) Transfected Lefty1 does not inhibit activity of exogenous recombinant TGFβ1; this experiment was performed similarly to that in panel (F). (H) The Lefty1(L300A/Q301A/L302A/P303A) point mutant (Mut) displays partial inhibitory activity relative to the wild-type (WT). The experiment was performed as in panel (C); the inset shows detection of wild-type and mutant Lefty1 protein expression. In all panels, assays were performed in triplicate; error bars represent one standard deviation.

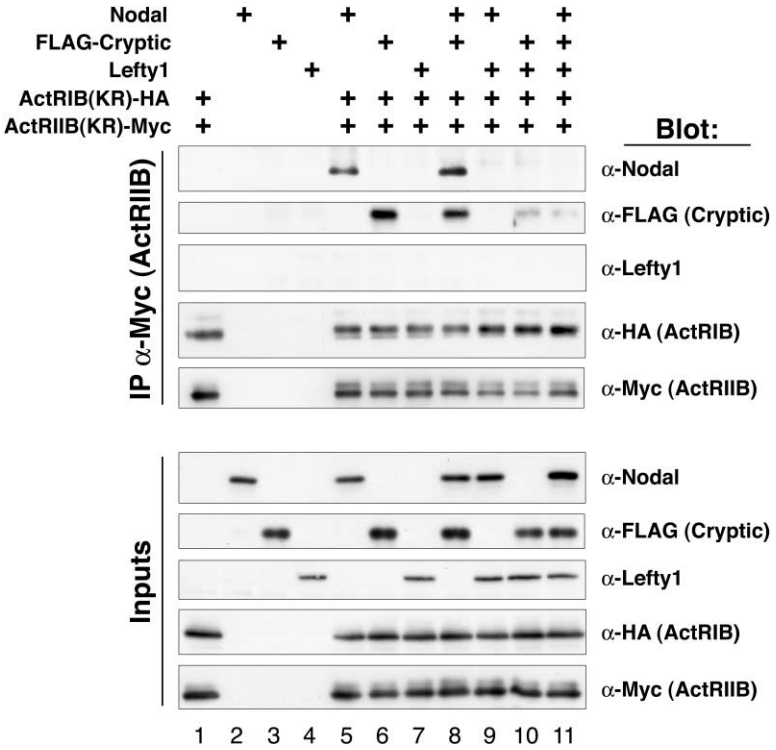


Figure 2. Two Independent Mechanisms for Lefty1 Inhibition of Nodal Receptor Complex Formation

Lefty1 expression inhibits Nodal interaction with activin receptors in the absence of EGF-CFC expression (lanes 5 and 9), as well as EGF-CFC interaction with activin receptors in the absence or presence of Nodal (lanes 6, 8, 10, and 11). Kinase-inactive activin receptor mutants were used for decreasing receptor internalization. The indicated expression constructs were cotransfected into 293T cells, followed by crosslinking with the membrane-impermeable reagent DTSSP, immunoprecipitation of epitope-tagged ActRIIB from cell lysates with anti-Myc antiserum, and reversal of crosslinking. Western blots of immunoprecipitated, and input proteins, detected with the following antibodies, are shown: α-Nodal (Nodal proprotein, approximately 38 kDa); α-FLAG (FLAG-Cryptic, approximately 22 kDa); α-Lefty1 (mature Lefty1, approximately 30 kDa); α-HA (HA-ActRIB, approximately 54 kDa); α-Myc (6xMyc-ActRIIB, approximately 75 kDa).

Nodal can form a complex with Cripto and the activin receptors ActRIB (ALK4) and ActRIIB in *Xenopus* animal caps [6]. To identify Nodal receptor complexes in mammalian cells, we expressed epitope-tagged proteins in 293T cells and used the membrane-impermeable reversible crosslinking reagent DTSSP for coimmunoprecipitation assays. For these experiments, we utilized the EGF-CFC protein Cryptic, which is required for left-right patterning in vivo [23]; we note that all of our signaling and protein interaction assays have yielded similar results with either Cripto or Cryptic to date ([8]; Figure 1; C.C., Y.-T. Yan, and M.M.S., unpublished data).

Our results indicate that Lefty can inhibit formation of a Nodal receptor complex through two distinct mechanisms. First, we observed that Nodal could interact with ActRIB and ActRIIB receptors in the absence of EGF-CFC protein expression and that this interaction could be blocked by Lefty1 (Figure 2, lanes 5 and 9). Interestingly, previous studies have reported that EGF-CFC proteins are required for Nodal to interact with activin receptors in *Xenopus* embryos [6, 19], although it has also been shown that Xnr1 can interact with the extracellular domain of ActRIIB in vitro [7]. These contrasting observations suggest that the interaction of Nodal with activin receptors may be kinetically unstable in the absence of EGF-CFC proteins and/or that there is an as-yet-uncharacterized protein or protein modification that inhibits this association in vivo but not in 293T cells.

Secondly, we found that Lefty1 could block the association between Cryptic and activin receptors, in either the absence or presence of Nodal (Figure 2, lanes 6, 8, 10, and 11). Notably, we did not detect any crosslinking of Lefty1 protein with activin receptors, in either the absence or presence of Nodal or EGF-CFC proteins. Thus, these results suggest two distinct mechanisms for Lefty inhibition of Nodal activity, through independent interactions of Lefty with Nodal and with EGF-CFC coreceptors.

### Interactions among Lefty, Nodal, and EGF-CFC Proteins

To investigate the basis by which Lefty protein can block Nodal receptor complex formation, we first examined whether Lefty could interact with Nodal in solution. To do this, we used culture supernatants from cells cotransfected with Lefty and Nodal expression constructs. We found that Lefty1 could immunoprecipitate Nodal in solution in the absence of crosslinking and that this association preferentially occurred with the mature Nodal protein (Figure 3A); similar results were obtained with Lefty2 (data not shown). In contrast, parallel experiments showed no interaction between Lefty1 and Activin (data not shown).

Next, we investigated whether Lefty protein could affect the interaction of Nodal with the EGF-CFC protein Cryptic in 293T cells. In the presence of increasing levels of Lefty1 protein, Nodal interaction with Cryptic progressively decreased, indicating that Lefty1 can compete with Nodal for the ability to bind to Cryptic (Figure 3B). Conversely, in the presence of increasing levels of Nodal protein, the association of Lefty1 with Cryptic steadily decreased, indicating that Nodal can compete with Lefty1 for the ability to interact with Cryptic (Figure 3C). In related experiments, we found that Lefty1 could also interact with Cripto and zebrafish Oep and that Lefty2 behaved similarly to Lefty1 in these assays (data not shown). Taken together, these results suggest that Nodal, Cryptic, and Lefty can each interact in a pair-wise man-

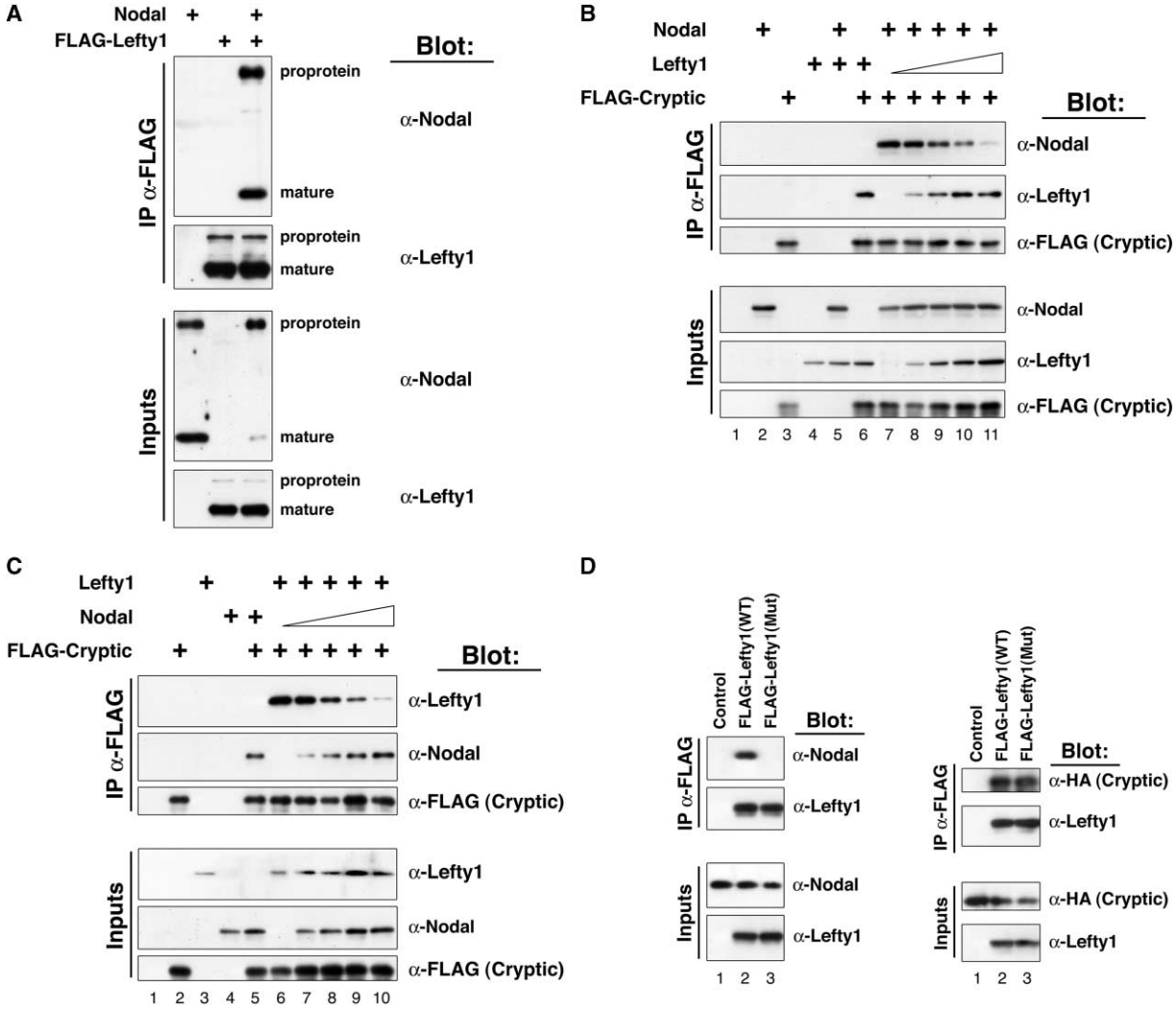


Figure 3. Interactions of Lefty1 with Nodal and Cryptic

(A) Nodal coimmunoprecipitates with Lefty1 in culture supernatants in the absence of crosslinking. Note that the mature form of Nodal is preferentially immunoprecipitated. Proteins were detected by Western blotting with the following antibodies: α-Nodal (Nodal proprotein, approximately 38 kDa; mature Nodal, approximately 13 kDa); α-FLAG (FLAG-Lefty1 proprotein, approximately 40 kDa; mature FLAG-Lefty1, approximately 30 kDa). (B) Increasing amounts of Lefty1 protein compete with Nodal for interaction with Cryptic. Expression constructs for the indicated proteins were cotransfected into 293T cells, followed by crosslinking with DTSSP, immunoprecipitation of epitope-tagged Cryptic from cell lysates with anti-FLAG antiserum, and reversal of crosslinking. Western blots of immunoprecipitated and input proteins, detected with the following antibodies, are shown: α-Nodal (Nodal proprotein, approximately 38 kDa); α-Lefty1 (mature Lefty1, approximately 30 kDa); α-FLAG (FLAG-Cryptic, approximately 22 kDa). (C) Increasing amounts of Nodal protein compete with Lefty1 for interaction with Cryptic. The experiment was performed similarly to that in panel (B). (D) The Lefty1(L300A/Q301A/L302A/P303A) point mutant interacts with Cryptic but not with Nodal. Experiments were performed similarly to those in panels (A) and (C).

ner but that the third protein can compete with this interaction; however, these experiments do not rule out the possibility that all three proteins can form a complex.

Finally, we generated a series of Lefty1 mutants in order to distinguish the abilities of Lefty to interact with Nodal and with Cryptic. For this purpose, we made point mutations in residues that may lie in a potential binding interface of the mature Lefty1 protein; we chose these residues based on sequence alignments with TGFβ1 and other TGFβ superfamily members [24, 25]. Although most of the Lefty point mutants generated were inactive as a result of their inability to be secreted or as a result of instability in culture supernatants (C.C. and M.M.S., unpublished data), we identified an alanine substitution mutant, Lefty1(L300A/Q301A/L302A/P303A), that has partial Nodal inhibitory activity in our cell culture assay (Figure 1H). Notably, this Lefty1 mutant is able to interact with Cryptic in coimmunoprecipitation assays but not with Nodal (Figure 3D). These data support the notion that the two modes of Lefty function are separable and can independently contribute to its inhibitory activity.

### Distinct Modes of Lefty Function Result in Stringent Regulation of Nodal Activity

The ability of Lefty proteins to inhibit Nodal signaling at two distinct steps may reflect differences in the context

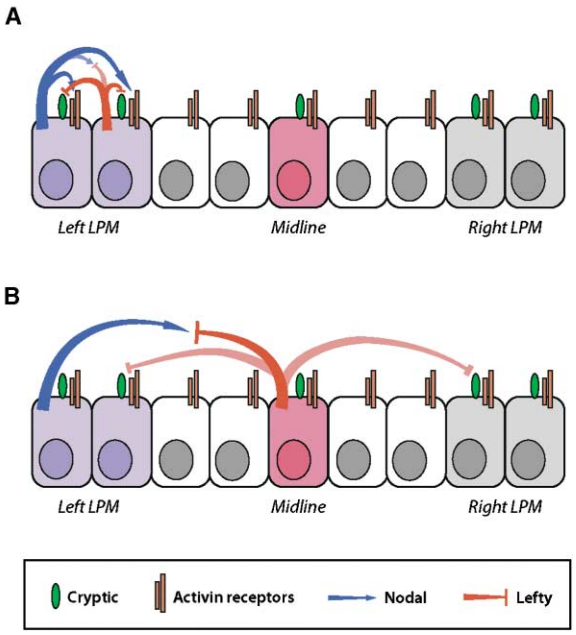


Figure 4. Conceptual Model for the Two Modes of Lefty Function in Inhibition of Nodal Signaling during Vertebrate Left-Right Patterning

Arrows are intended to show flow of activity and do not presume a specific mechanism of protein propagation. *Nodal*- and *Lefty*-expressing cells in the left lateral-plate mesoderm (LPM) at early somite stages are shown in purple, whereas *Lefty*-expressing cells in the axial midline are shown in red. (A) In the left lateral-plate mesoderm, where *Lefty* and *Nodal* are coexpressed, Lefty proteins can function efficiently and specifically by inhibiting EGF-CFC coreceptor activity (dark red) or by interacting with Nodal itself (light blue/red). (B) *Lefty* expression in the axial midline can block long-range Nodal activity emanating from the left lateral-plate mesoderm through an inhibitory interaction with Nodal (dark blue/red) or perhaps through interaction with EGF-CFC proteins in the lateral-plate mesoderm (light red).

in which Lefty functions in vivo, particularly during left-right patterning. In all vertebrates examined thus far, Lefty expression occurs in both the left lateral plate mesoderm, where it is coexpressed with an EGF-CFC protein and a Nodal-related protein, and the axial midline, where it is coexpressed with an EGF-CFC protein but not with Nodal (Figure 4) [14, 15, 26]. In the mouse, *Lefty1* expression in the prospective floor plate has been proposed to function as a "molecular barrier" for long-range propagation of Nodal signals to the anterior right lateral-plate mesoderm [27]. In contrast, *Lefty2* expression in the left lateral-plate mesoderm is believed to antagonize the left-sided positive feedback loop for *Nodal*, as well as to prevent excess Nodal activity from overflowing to the posterior right lateral plate mesoderm [17].

We propose that these functions for *Lefty* genes in vivo can be mediated at least in part by the different mechanisms of Lefty inhibitory activity that we have identified. Thus, in the left lateral-plate mesoderm, where *Lefty* and *Nodal* are coexpressed, Lefty proteins could antagonize Nodal signaling specifically and efficiently by blocking EGF-CFC coreceptor activity and perhaps by interacting with Nodal itself (Figure 4A). In contrast, when Nodal can potentially signal at long-range in vivo [17, 28], such as from the left lateral-plate mesoderm toward the axial midline, Lefty inhibitory activity could be mediated by a direct interaction with Nodal itself (Figure 4B). In principle, the two modes of Lefty activity might be particularly relevant in situations where Lefty can potentially act as a long-range factor [11–13, 17]; our model would also be consistent with inhibitory interactions of Lefty with EGF-CFC proteins in the lateral-plate mesoderm.

Our model for Lefty inhibition of Nodal signaling through interaction with EGF-CFC coreceptors partially resembles that proposed for the transmembrane protein Tomoregulin, which can also antagonize Nodal signaling through interaction with Cripto, although in a cell-autonomous manner [29]. It is also formally analogous to the proposed mode of action of Dickkopf (Dkk) proteins in inhibiting canonical Wnt signaling mediated by LRP5/6 coreceptors [30]. However, superimposed upon this general mechanism is an additional level of inhibition provided by the association of Lefty with Nodal ligand. This inhibition may correspond to a direct interaction or may instead reflect an indirect interaction with as-yet-uncharacterized associated proteins. We note that this interaction may impose additional specificity to Lefty inhibitory activity and that this specificity may not necessarily correspond to that conferred by interaction with EGF-CFC proteins.

This analysis of the molecular properties of Lefty proteins may also provide insight into situations, as yet poorly characterized, in which *Lefty* expression occurs in the apparent absence of Nodal signaling. For example, *LEFTY1* (*LEFTYB*) is expressed in human colon crypts [31], and there is currently no evidence that Nodal is expressed in this tissue. Our finding that Lefty can antagonize Vg1 signaling suggests that Lefty proteins could also inhibit other TGFβ superfamily members in vivo, at least those requiring EGF-CFC function for their signaling activity.

In summary, our analysis of Lefty function at the molecular level reveals a hitherto unappreciated complexity to its inhibitory activity. We propose that the distinct and independent mechanisms of Lefty function provide an additional level of specificity and stringency for regulation of Nodal activity. Such a dual mode of inhibitory function may be highly advantageous for the tight regulation of a potent morphogenetic factor.

## Experimental Procedures

### Expression Constructs

The FLAG-tagged mouse *pcDNA3-Lefty1* expression construct was generated by PCR from cDNA templates [15], with the epitope tag inserted after the second proprotein convertase cleavage site. FLAG-tagged *Lefty1* point mutants were generated by standard methods and confirmed by sequencing. The *pcDNA3-BMP2-Vg1* expression construct was derived from *pSP64T3-BMP2-Vg1* [32], and the kinase-inactive mutants ActRIIB(K217R)-Myc and ActRIB(K234R)-HA were kindly provided by M. Whitman [6]. Other plasmids for luciferase reporter assays were described previously [8].

### Cell Culture Assay for Nodal and Lefty Activities

Luciferase assays for Nodal activity were performed as previously described [8]; relative activities represent the average of experiments performed in triplicate. Expression levels of Lefty1 protein

were detected with a polyclonal antiserum to mouse Lefty1; this antiserum was generated with a glutathione-S-transferase (GST) fusion with and the mature region of Lefty1 as an immunogen (Cocalico Biologicals). Expression levels of epitope-tagged EGF-CFC proteins were detected with a monoclonal anti-FLAG M2 antibody (Sigma). When used, recombinant human Activin A (100 pM), TGFβ1 (500 pM), and Follistatin (1 nM), all from R&D Systems, were added to the culture medium 8 hr after transfection. Nodal-conditioned medium was collected from a stable 293T clone (#9) [8]; Lefty1-conditioned medium was collected from 293T cells transiently transfected with *pcDNA3-FLAG-Lefty1*.

### Protein Crosslinking and Coimmunoprecipitation Analysis

Reversible chemical crosslinking and coimmunoprecipitation were carried out as described previously [8], except that intact cells were incubated in culture medium containing 0.5 mM DTSSP (3,3′-dithiobis[sulfosuccinimidyl proprionate]) (Pierce) at 4°C for 2 hr. The reaction was subsequently stopped with 50 mM Tris-Cl. For detection of the association of Lefty1 and Nodal in culture supernatants, 293T cells were transfected with *pcDNA3-Nodal* and *pcDNA3-FLAG-Lefty1*; culture supernatants were collected 48 hr after transfection and directly subjected to immunoprecipitation with anti-FLAG M2 antibody (Sigma). For all other experiments, crosslinking with DTSSP was carried out prior to coimmunoprecipitation, and whole-cell lysates prepared with RIPA114 buffer as described were used [8]. Western blotting was performed with the antibodies described above, as well as anti-HA monoclonal antibodies (Covance) and a polyclonal antiserum against mouse Nodal [8].

### Acknowledgments

We thank Yu-Ting Yan for assistance and insights at early stages of this project, Dan Kessler, Hiroshi Hamada, and Malcolm Whitman for generously providing plasmids, and Cory Abate-Shen and Raymond Habas for helpful discussions and comments on the manuscript. This work was supported by National Institutes of Health grants HD38766 and HD42837.

Received: December 10, 2003
Revised: February 4, 2004
Accepted: February 18, 2004
Published online: February 26, 2004

### References

1. Schier, A.F., and Shen, M.M. (2000). Nodal signalling in vertebrate development. Nature *403*, 385–389.
2. Whitman, M. (2001). Nodal signaling in early vertebrate embryos. Themes and variations. Dev. Cell *1*, 605–617.
3. Schier, A.F. (2003). Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. *19*, 589–621.
4. Kumar, A., Novoselov, V., Celeste, A.J., Wolfman, N.M., ten Dijke, P., and Kuehn, M.R. (2001). Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J. Biol. Chem. *276*, 656–661.
5. Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C.Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M., et al. (2000). Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. Mol. Cell *5*, 35–47.
6. Yeo, C., and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol. Cell *7*, 949–957.
7. Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M.G., Ibanez, C.F., and Brivanlou, A.H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. Genes Dev. *15*, 2010–2022.
8. Yan, Y.T., Liu, J.J., Luo, Y., E.C., Haltiwanger, R.S., Abate-Shen, C., and Shen, M.M. (2002). Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol. Cell. Biol. *22*, 4439–4449.
9. Thisse, C., and Thisse, B. (1999). Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. Development *126*, 229–240.
10. Cheng, A.M., Thisse, B., Thisse, C., and Wright, C.V.E. (2000). The lefty-related factor Xatv acts as a feedback inhibitor of nodal signaling in mesoderm induction and L-R axis development in Xenopus. Development *127*, 1049–1061.
11. Chen, Y., and Schier, A.F. (2002). Lefty proteins are long-range inhibitors of squint-mediated nodal signaling. Curr. Biol. *12*, 2124–2128.
12. Sakuma, R., Ohnishi Yi, Y., Meno, C., Fujii, H., Juan, H., Takeuchi, J., Ogura, T., Li, E., Miyazono, K., and Hamada, H. (2002). Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion. Genes Cells *7*, 401–412.
13. Branford, W.W., and Yost, H.J. (2002). Lefty-dependent inhibition of nodal- and wnt-responsive organizer gene expression is essential for normal gastrulation. Curr. Biol. *12*, 2136–2141.
14. Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y., and Hamada, H. (1996). Left-right asymmetric expression of the TGF beta-family member lefty in mouse embryos. Nature *381*, 151–155.
15. Meno, C., Ito, Y., Saijoh, Y., Matsuda, Y., Tashiro, K., Kuhara, S., and Hamada, H. (1997). Two closely-related left-right asymmetrically expressed genes, lefty-1 and lefty-2: their distinct expression domains, chromosomal linkage and direct neuralizing activity in Xenopus embryos. Genes Cells *2*, 513–524.
16. Iratni, R., Yan, Y.T., Chen, C., Ding, J., Zhang, Y., Price, S.M., Reinberg, D., and Shen, M.M. (2002). Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. Science *298*, 1996–1999.
17. Meno, C., Takeuchi, J., Sakuma, R., Koshiba-Takeuchi, K., Ohishi, S., Saijoh, Y., Miyazaki, J., ten Dijke, P., Ogura, T., and Hamada, H. (2001). Diffusion of nodal signaling activity in the absence of the feedback inhibitor Lefty2. Dev. Cell *1*, 127–138.
18. Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., and Schier, A.F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell *97*, 121–132.
19. Cheng, S.K., Olale, F., Bennett, J.T., Brivanlou, A.H., and Schier, A.F. (2003). EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. Genes Dev. *17*, 31–36.
20. Thisse, B., Wright, C.V., and Thisse, C. (2000). Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. Nature *403*, 425–428.
21. Tanegashima, K., Yokota, C., Takahashi, S., and Asashima, M. (2000). Expression cloning of Xantivin, a Xenopus lefty/antivin-related gene, involved in the regulation of activin signaling during mesoderm induction. Mech. Dev. *99*, 3–14.
22. Branford, W.W., Essner, J.J., and Yost, H.J. (2000). Regulation of gut and heart left-right asymmetry by context-dependent interactions between xenopus lefty and BMP4 signaling. Dev. Biol. *223*, 291–306.
23. Yan, Y.-T., Gritsman, K., Ding, J., Burdine, R.D., Corrales, J.D., Price, S.M., Talbot, W.S., Schier, A.F., and Shen, M.M. (1999). Conserved requirement for EGF-CFC genes in vertebrate left-right axis formation. Genes Dev. *13*, 2527–2537.
24. Newfeld, S.J., Wisotzkey, R.G., and Kumar, S. (1999). Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics *152*, 783–795.
25. Innis, C.A., Shi, J., and Blundell, T.L. (2000). Evolutionary trace analysis of TGF-beta and related growth factors: implications for site-directed mutagenesis. Protein Eng. *13*, 839–847.
26. Shen, M.M., Wang, H., and Leder, P. (1997). A differential display strategy identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development *124*, 429–442.
27. Meno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Ohishi, S., Noji, S., Kondoh, H., and Hamada, H. (1998). lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. Cell *94*, 287–297.
28. Chen, Y., and Schier, A.F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. Nature *411*, 607–610.
29. Harms, P.W., and Chang, C. (2003). Tomoregulin-1 (TMEFF1)

inhibits nodal signaling through direct binding to the nodal coreceptor Cripto. Genes Dev. *17*, 2624–2629.
30. Moon, R.T., Bowerman, B., Boutros, M., and Perrimon, N. (2002). The promise and perils of Wnt signaling through beta-catenin. Science *296*, 1644–1646.
31. Yashiro, K., Saijoh, Y., Sakuma, R., Tada, M., Tomita, N., Amano, K., Matsuda, Y., Monden, M., Okada, S., and Hamada, H. (2000). Distinct transcriptional regulation and phylogenetic divergence of human LEFTY genes. Genes Cells *5*, 343–357.
32. Thomsen, G.H., and Melton, D.A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. Cell *74*, 433–441.